A Research Note

Inhibition of *Clostridium botulinum* in Comminuted Bacon by Short-Chain Alkynoic and Alkenoic Acids and Esters

ABSTRACT

Several short-chain alkynoic and alkenoic acids and esters were screened for inhibition of gas production by *Clostridium botulinum* in cans of comminuted, nitrite-free, temperature-abused bacon. The most active compounds were propiolic (2-propynoic) acid, methyl and ethyl propiolate, 2-propenoic acid, methyl and ethyl propenoate, and mono- and dimethyl and ethyl fumarate. Maleic acid, methylmaleic acid, ethyl maleate, 2-methyl propenoic acid, *trans*-2-methyl crotonic acid, 3-methyl-2-butenoic acid, 3-methylallyl alcohol, vinyl crotonate, methylmalonic acid, dimethyl glutarate, 2,4-hexadien-1-ol, *cis*-3-hexenoic acid, 4-pentenoic acid, *trans*-2-pentenoic acid and ethylidene acetic acid were less active. A more comprehensive study on the inhibition of toxin formation by *C. botulinum* in comminuted nitrite-free bacon was done by comparing equimolar quantities of some of these compounds with $NaNO_2$ at 120 μg/g and sorbic acid at 9 and 18 mM (0.10 and 0.20%). These studies showed that propiolic acid was more effective than either nitrite or sorbic acid. Compounds in this study less active than nitrite or sorbic acid were 3-methylallyl alcohol, 2,4-hexadien-1-ol, dimethyl glutarate and methylmaleic acid.

The potential for botulinal toxin formation in bacon if nitrite is omitted has prompted considerable research into identifying potential substitutes for inhibiting *Clostridium botulinum*. Many possible alternatives have been suggested (*7*). Methyl and ethyl esters of fumaric acid have been shown to be inhibitory to *C. botulinum* in a model bacon system (*4*); the present study was intended to determine the antibotulinal activity of compounds closely related to the fumarate esters.

[1]*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.*

MATERIALS AND METHODS

Preparation of bacon

The test system used in these screening studies was nitrite-free bacon prepared under commercial conditions. After processing, the bacon was comminuted and mixed to provide a uniform medium for studies on the effect of various chemicals on growth of *C. botulinum*.

Nitrite-free bacon was prepared by a local bacon processor using a brine that contained 11.4% salt, 2% sodium tripolyphosphate, 0.35% sodium erythorbate, 3.2% sucrose and 0.21% hydrolyzed plant protein. Pumping (10%) and processing were designed to bring the finished bacon back to green weight. After processing, the bacon was frozen and ground through a 0.188-in. (0.476-cm) plate in a Hobart cutter[1] (model 84-145) and mixed in a Buffalo food chopper (model B52). Two-kilogram quantities were vacuum sealed in plastic pouches and kept frozen until needed. Analyses were by methods of the Association of Official Analytical Chemists (*2*).

Clostridium botulinum *spores*

Nineteen strains of *C. botulinum*, ten of type A and nine of type B, were cultured and spore crops prepared as described previously (*6*). The strains of type A were obtained from the Centers for Disease Control, Atlanta, GA (No. 20PLALC); Food and Drug Administration, Washington, DC (Nos. 3, 78, 429, 62, 69, 426); U.S. Army Laboratories, Natick, MA (No. 33); American Type Culture Collection (ATCC), Rockville, MD (No. 25763); and Northern Regional Research Laboratories, Peoria, IL (No. B1218). The strains of type B were obtained from the Centers for Disease Control (No. 17409); Food and Drug Administration (Nos. 383, 999, 8688R, 642, 169); U.S. Army Laboratories (No. 53); ATCC (No. 7949); and Animal and Plant Health Inspection Service, USDA, Beltsville, MD (No. 770). Equal amounts of spores from each strain were combined to give a mixture containing 2.8×10^5 spores per ml. The mixture was heated at 68°C for 30 min and was stored in a refrigerator.

Spore counts of inoculated meat

Spore counts were made by a 3-tube most probable number method. Meat (40 g) was blended (Stomacher R) in 360 ml of freshly autoclaved fluid thioglycollate medium (Difco). Three 10-ml portions were placed in sterile 16 × 125-mm tubes and three 1-ml portions were mixed with 9 ml of thioglycollate medium in tubes. The original blend was serially diluted three times with thioglycollate medium and three portions of 10 ml each were added to tubes. The dilutions represented 1, 0.1, 0.01, 0.001 and 0.0001 g of the original meat. The tubes were incubated 5 d at 33°C in an anaerobic incubator (National Appliance Co.) with N_2 replacement following evacuation to 22 in. vacuum. After incubation, centrifuged supernatant fluids (3000 × *g*, 15 min) of the tubes' contents were tested for toxin.

Determination of antibotulinal activity

Following thawing in running cold tap water, the bacon was thinly spread out (1 cm thick) and additions of *C. botulinum* spores and inhibitors were made by pipetting (if liquid) or sprinkling (if solid). The bacon was then mixed using gloved hands. After thorough mixing, the material was placed in three 208 × 107 aluminum tab cans that were sealed under vacuum, heated to 68°C (center temperature) for 30 min to activate the spores and inactivate possible competing vegetative cells, cooled in tap water, and incubated at 30°C. Lack of gas production, as shown by failure of cans to swell, was used as the presumptive indicator of antibotulinal activity in the screening procedure.

A more comprehensive test for activity was done using 15 cans of bacon for each treatment. Five cans were removed after 2 wk of incubation, five after 4 wk, and five after 8 wk. Cans were removed earlier if swollen.

Toxin testing

Toxin was determined by injecting (i.p.) pairs of mice with gelatin phosphate extracts and observing for typical respiratory symptoms of botulism (*6*). Heated extracts (100°C, 10 min) and neutralization with specific antisera (1.2 ml extract and 0.3 ml antiserum incubated 37°C, 1 h) confirmed that samples were positive or negative for botulinal toxin. The compounds used in these studies were obtained from Pfaltz and Bauer, Aldrich Chemicals, or Alfa Products.

RESULTS AND DISCUSSION

The comminuted bacon contained 35.9% moisture and 2.07% NaCl (5.45% moisture-phase NaCl). This concentration of NaCl was higher than the target level of 1.5%; however, it has been shown that attainment of target levels of pumping brine is difficult to achieve in all bellies (*6*). The amount of NaCl in the moisture phase (5.45%) was insufficient to cause significant inhibition of *C. botulinum (8)* unless nitrite was also added. Results of the antibotulinal screening tests are shown in Table 1. Spore counts of the inoculated samples were not determined for these tests. Each compound was compared to control samples and to meat containing 120 μg of added $NaNO_2$/g. At an initial concentration of 0.150%, the following compounds were comparable in activity to $NaNO_2$: methylmaleic acid, 2-propenoic (acrylic) acid, 2-methyl propenoic acid, methyl and ethyl propenoate, propiolic acid (2-propynoic), methyl and ethyl propiolate, 3-methyl-2-butenoic acid, 3-methylallyl alcohol, and the methyl and ethyl esters of fumaric acid. When retested at 0.075%, the only compounds equal in activity to $NaNO_2$ were : 2-propenoic acid, propiolic acid and ethyl propiolate. At concentrations of 0.075%, compounds less active than $NaNO_2$ were 2-methyl propenoic acid, ethyl-2-propenoate, methyl-2-propenoate and methyl propiolate. Propiolic acid at 0.038% was as active as $NaNO_2$, whereas the other compounds at this level had little or no activity.

Compounds having no activity at the 0.15% level were: potassium sorbate, ethyl-2,4-hexadienoate, methyl-2-hexenoate, methyl-2,4-hexadienoate, *trans*- 2-hexenoic acid, *cis*-3-hexenoic acid, ethyl-3-hexenoate, ethyl-2-hexenoate, tiglic acid, ethyl tiglate, 2-methyl-2-pentenoic acid, 1-penten-3-ol, 4-penten-1-ol, methyl crotonate, ethyl crotonate, diethyl methylmalonate, ethyl malonate, itaconic acid, glutaconic acid, diethyl glutaconate, dimethyl succinate, fumaric acid, methyl fumaric acid, dibutyl fumarate, bis (2-ethylhexyl) fumarate, dihydroxy fumaric acid, diethyl maleate, dihydroxy malic acid, methyl maleate, ethyl maleate, dibutyl maleate, bis (2-ethylhexyl) maleate, diethyl malonic acid, methyl malonate, dimethyl malonate, diethyl ethylidenemalonate and diethyl allylmalonate.

It is interesting that among the inactive or slightly active compounds were the six-carbon compounds with two double bonds, including potassium sorbate and its methyl and ethyl esters. Six-carbon compounds with only one double bond were also inactive. Although methylmalonic acid was slightly active, none of the other malonic acid derivatives had activity. Methyl fumaric acid, dibutyl fumarate, 2-ethylhexyl fumarate and dihydroxy fumaric acid did not inhibit *C. botulinum* under these conditions, although previous work (*4*) had shown that the methyl and ethyl esters of fumaric were active. Ethyl, methyl and diethyl esters of malic were also inactive.

Results of the more comprehensive test for antibotulinal activity, using equimolar concentrations of compounds, are in Table 2. The control cans all became swollen before the initial 2-wk removal period, and all were toxic. With 120 μg $NaNO_2$/g, none of the cans was swollen or toxic at the end of 4 wk, but all were toxic at the end of 8 wk. When bacon was made with sorbic acid at 9 mM concentrations (0.10%), the cans were swollen and toxic before 4 wk, but at 18 mM there were no toxic cans until 8 wk. This is in agreement with previous results on sorbic acid inhibition of *C. botulinum* toxin formation (*6*) when sorbic acid was added to the curing pickle. Sorbic alcohol (2,4-hexadien-1-ol) had no antibotulinal activity, even at the 18-mM level. Propiolic acid was the most active compound tested, preventing toxin formation completely for the duration of the 8-wk abuse period.

3-methylallyl alcohol had some activity for up to 2 wk, but by 4 wk all cans were toxic. Dimethyl glutarate and methylmaleic acid at 18 mM concentrations also had some activity for up to 2 wk, but by 2 wk all cans containing these compounds were toxic.

The data in Table 2 indicate that toxicity preceded can swelling. This observation has been made by others working with meat systems inoculated with *C. botulinum* spores (*6,9*). However, the data further indicate that toxic cans usually produce enough gas to cause swelling. In this test, all cans that were swollen were also toxic; can swelling is, therefore, useful as a presumptive test for growth of *C. botulinum* in meat systems, but it cannot be considered to be the earliest or absolute indicator of toxicity.

The results of the spore counts on the meat containing 18-mM levels of the compounds before and after heating, were (spores per g): control (900, 400); $NaNO_2$ (450, 300); 3-methylallyl alcohol (280, 690); 2,4-hexadien-1-ol

TABLE 1. *Alkynoic and alkenoic acids and esters inhibitory to* C. botulinum *in comminuted nitrite-free bacon*[a].

Test compound	Concentration (%)	Days to first swell and No. of cans swollen[b]					
		Control	No.	Test compound	No.	120 μg nitrite/g	No.
Maleic acid	0.150	7	3	24	1	55	1
	0.100	6	3	7	3	41	3
Methylmaleic acid	0.150	7	3	>60	0	55	1
	0.075	8	3	10	3	>60	0
2-Propenoic acid	0.150	11	3	>60	0	60	1
	0.075	7	3	>60	0	55	1
	0.038	8	3	27	1	>60	0
2-Methyl propenoic acid	0.150	11	3	>60	0	60	1
	0.075	8	3	24	3	>60	0
	0.038	8	3	12	3	>60	0
Ethyl-2-propenoate	0.150	11	3	>60	0	60	1
	0.075	7	3	43	2	55	1
	0.038	8	3	12	3	>60	0
Methyl-2-propenoate	0.150	11	3	>60	0	60	1
	0.075	7	3	20	3	55	1
Propiolic acid	0.150	9	3	>60	0	57	1
	0.075	8	3	>60	0	>60	0
	0.038	8	3	>60	0	>60	0
Methyl propiolate	0.150	9	3	>60	0	57	1
	0.075	8	3	21	2	>60	0
	0.038	8	3	21	3	>60	0
Ethyl propiolate	0.150	7	3	>60	0	55	1
	0.075	7	3	>60	0	55	1
	0.038	8	3	14	3	>60	0
trans-2-Methyl crotonic acid	0.150	11	3	36	3	60	1
	0.075	7	3	12	3	51	2
3-Methyl-2-butenoic acid	0.150	11	3	>60	0	60	1
	0.075	7	3	14	3	51	2
3-Methyllallyl alcohol	0.150	11	3	57	1	60	1
	0.075	7	3	12	3	51	2
Ethyl maleate	0.150	11	3	29	2	60	1
Vinyl crotonate	0.150	9	3	26	3	49	1
Methylmalonic acid	0.150	11	3	24	3	60	1
Dimethyl glutarate	0.150	11	3	16	3	60	1
2,4-Hexadien-1-ol	0.150	12	3	33	3	>60	0
cis-3-Hexenoic acid	0.150	12	3	21	3	>60	0
4-Pentenoic acid	0.150	12	3	20	3	>60	0
trans-2-Pentenoic acid	0.150	12	3	19	3	>60	0
Ethylidene acetic acid	0.150	12	3	18	3	>60	0
Monomethyl fumarate	0.150	7	3	>60	0	46	3
	0.075	7	3	15	3	46	3
Dimethyl fumarate	0.150	7	3	>60	0	46	3
	0.075	7	3	10	3	46	3
Monoethyl fumarate	0.150	7	3	>60	0	46	3
	0.075	7	3	4	2	46	3
Diethyl fumarate	0.150	7	3	>60	0	46	3
	0.075	7	3	8	3	46	3
Potassium sorbate	0.150	7	3	7	3	46	3

[a]Several separate experiments were done; the results for each compound are given with its own controls.
[b]Three cans were tested for each treatment.

TABLE 2. *Inhibition of* Clostridium botulinum *toxin production in bacon by selected acids and esters.*

Compound	pH	Concentration (mM)	No. of cans toxic when removed[a] at: 2 wk	4 wk	8 wk
None	6.6	—	15/15 (15)[b]	—	—
$NaNO_2$	6.5	1.74 (120 μg/g)	0/5	0/5	5/5 (2)
3-Methylallyl alcohol	6.5	9	0/5	5/5 (3)	5/5 (5)
3-Methylallyl alcohol	6.4	18	1/5	5/5	5/5 (5)
2-4-Hexadien-1-ol	6.5	9	5/5	10/10 (9)	—
2-4-Hexadien-1-ol	6.4	18	3/5	5/5 (1)	5/5 (5)
Dimethyl glutarate	6.2	9	5/5 (1)	10/10 (10)	—
Dimethyl glutarate	5.8	18	0/5	4/5	3/5 (1)
Methylmaleic acid	6.1	9	6/6 (6)	9/9 (9)	—
Methylmaleic acid	6.0	18	0/5	5/5 (3)	5/5 (5)
Sorbic acid	6.4	9 (0.10%)	5/5	10/10 (10)	—
Sorbic acid	6.2	18 (0.20%)	0/5	0/5	2/5
Propiolic acid	6.3	9	0/5	0/5	0/5
Propiolic acid	6.2	18	0/5	0/5	0/5

[a]15 cans each treatment; five were removed after 2, 4 or 8 wk or earlier if swollen.
[b]Number in parentheses is number of swollen cans.

(120, 700); dimethyl glutarate (390, 590); methyl maleic acid (190, 380); sorbic acid (500, 600); and propiolic acid (450, 350). Although widely disparate, these figures are within the 95% confidence interval for the MPN method and on average gave a spore count of 450/g (410 before, 450 after heating).

There is little information in the literature regarding the antimicrobial properties of aliphatic compounds with double or triple bonds. Ethyl malonate, at a concentration of 0.02%, inhibited the cellulase enzyme of *Heterocephalum aurantiacum (1)*. This compound was suggested as a possible antifungal agent in preventing deterioration of textiles. Another report (*3*) indicated that 0.1% diethyl malonate was not inhibitory to saprophytic aquatic bacteria but did inhibit infusorians. The antibotulinal activity of the fumarate esters in bacon was recently reported (*4*). At levels of 0.125%, the methyl and ethyl esters were as active as 120 μg $NaNO_2$/g.

Any possible food application for these compounds would require knowledge of their effects on higher organisms, but unfortunately little or no toxicity data are available. Propenoic acid and its methyl and ethyl esters, and maleic acid are irritants. The oral rat LD_{50} of methylmaleic acid according to the *Registry of Toxic Effects of Chemical Substances* (Dept. of Health, Education and Welfare) is 1320 mg/kg; methylmalonic acid is listed in the Registry as having a lowest reported toxic level (LDL_o) of 500 mg/kg when injected intraperitoneally. The Registry also lists oral rat LD_{50}'s of 610 mg/kg for *trans*-2-pentenoic acid, 2140 mg/kg for 2,4-hexadien-1-ol (sorbic alcohol), and 470 mg/kg for allylacetic acid. The irritant properties of the acrylic acid derivatives appear to rule them out as potential food additives; however, methylmaleic acid, which is prepared by heating citric acid, may have some application. No information is available on the toxicity of the most active compound, propiolic acid.

The compounds reported here do not represent an exhaustive study of the antibotulinal properties of the short-chain alkynoic or alkenoic acids or esters. It is possible that others have similar inhibitory properties and are less toxic. The literature data on toxicity is inadequate for immediate consideration of these compounds as food additives because extensive (and expensive) toxicity studies would be required before such use could be contemplated. Their effect on the organoleptic and physical properties of foods also would have to be assessed. However, the data indicate that these compounds, especially propiolic acid and its esters, are worthy of further study as inhibitors of *C. botulinum* and other microorganisms.

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